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Application No.	
Date of Filing	22nd March 2002
Applicant	University College Dublin, National University of Ireland, Dublin, established by charter dated 1908 of Belfield, Dublin 4, Ireland

PRIORITY DOCUMENT
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Dated this 9th day of April 2003.

Brian McKernan

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FORM NO. 1

S 020209

Application No.

REQUEST FOR THE GRANT OF A PATENT
PATENTS ACT, 1992

The Applicant named herein hereby request

the grant of a patent under Part II of the Act

X the grant of a short-term patent under Part III of the Act

on the basis of the information furnished hereunder.

1. **APPLICANT**

Name	**University College Dublin, National University of Ireland, Dublin**
Address	**Belfield Dublin 4 Ireland**
Description/Nationality	**established by charter dated 1908.**

2. **TITLE OF INVENTION**

"Compounds useful as photodynamic therapeutic agents"

3. **DECLARATION OF PRIORITY ON BASIS OF PREVIOUSLY FILED APPLICATION FOR SAME INVENTION (SECTIONS 25 & 26)**

Previous filing date	Country in or for which filed	Filing No.
N/A		

4. **IDENTIFICATION OF INVENTOR(S)**
Name(s) of person(s) believed by Applicant(s) to be the inventor(s)
1. Donal F. O'Shea
2. John Killoran
3. William Gallagher

Address
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5. **STATEMENT OF RIGHT TO BE GRANTED A PATENT (SECTION 17(2)(B))**

By virtue of Assignment dated March 21, 2002

Contd./...

Compounds useful as Photodynamic Therapeutic Agents

The present invention relates to compounds useful as photodynamic therapeutic agents. The present invention also relates to methods of photodynamic therapy, by administration of the said compounds.

Photodynamic therapy (PDT) is a non-invasive technique for the treatment of a variety of solid tumour types by the combined use of visible or near-visible light with a photosensitising drug. PDT also has application in certain non-neoplastic diseases including age-related macular degeneration and periodontal diseases caused by overgrowth of pathogenic microflora around the teeth. The therapeutic strategy is as follows: A photosensitiser of low dark toxicity is introduced into the body, which photosensitiser accumulates preferentially to some extent within the tumour. The tumour is then irradiated with low energy light through the body's therapeutic window, i.e. beyond the absorbance of body tissue, (5[illegible]800 nm), resulting in excitation of the photosensitiser. The light activated photosensitiser can then transfer its excited state energy to surrounding biological tissue through molecular oxygen, resulting in oxidative cellular damage leading to cell death *via* apoptosis and/or necrosis. After light treatment, the photosensitiser is allowed to clear from the body. PDT can be viewed as a highly selective form of cancer treatment, provided that the photosensitiser is non-toxic in the absence of light, so that only the irradiated areas are affected.

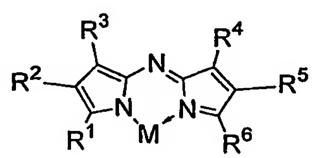
Most known PDT compounds investigated to date are based on cyclic-tetrapyrrole macrocycles, from which it can be difficult to generate a range of sequentially modified derivatives (M. Wainwright, *Chem. Soc. Rev.*, 1996, 351).

At the present time, Photofrin®, a haematoporphyrin derivative, is the only clinically available PDT agent. It has been approved for use in the United States, Japan and Europe for the treatment of oesophageal, lung, stomach, and cervical

cancers (R. Bonnett, *Chem. Soc. Rev.*, 1995, **24**, 19. and T.J. Dougherty, C.J. Gomer, B.W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan, and Q. Peng, *J. Natl. Cancer Inst.* 1998, **90**, 889). Although it is the sole approved anti-cancer PDT agent, it is widely recognised that it is far from being an ideal drug for use in PDT (I.J. MacDonald and T.J. Dougherty, *J. Porphyrins Phthalocyanines*, 2001, 5, 105). Despite its achievements to date, PDT is still in its developmental stages, with there being a marked need to develop improved photosensitisers with better efficacy and side effect profiles. In order to further advance this novel form of treatment, it has become apparent that the development of new PDT compounds, together with a more thorough and integrated understanding of the multitude of targets/actions so far ascribed to PDT agents, is needed.

The present invention solves the problems of the prior art by providing synthesis, photophysical properties and *in vitro* cellular uptake evaluation of a new class of potential PDT agent, derived from azadipyrromethenes whose tetraaryl derivatives 1 which were first reported in 1940's but which, since then, have remained relatively unstudied (M.A.T. Rogers, *J. Chem. Soc.*, 1943, 596 and E.B. Knott, *J. Chem. Soc.*, 1947, 1196).

The present invention, therefore, provides a compound of the formula



in which M is a chelating agent; R^1, R^2, R^3, R^4, R^5 and R^6 can each, independently, be H; a substituted or unsubstituted, saturated or unsaturated, cyclic, preferably aryl moiety; a substituted or unsubstituted, saturated or unsaturated heterocyclic, preferably heteroaryl moiety; or a substituted or unsubstituted, saturated or unsaturated, straight or branched chain alkyl moiety and R^2 and R^5 can each, in addition and independently, be a heavy atom,

preferably a halogen selected from At, I, Br or Cl, of which I or Br is most preferred. The present invention also provides salts of the aforementioned compounds.

Preferably, M is BX_2, in which each X is, independently, a halide. Most preferably, each halide is a fluoride. Alternatively, M is a metal selected, preferably, from Zn, Al, Si, Mg, Lu and Sn.

As used herein, the term "heavy atom" is intended to embrace atoms with an atomic weight greater than 30. Selenium is another example of a heavy atom.

As used herein, the term "cyclic" is intended to embrace substituted or unsubstituted, saturated or unsaturated, moieties containing one or more rings. If more than one ring is present, the rings may be fused together. Suitable are substituted or unsubstituted steroids.

As used herein, the term "aryl", which is included within the scope of "cyclic", is intended to embrace substituted or unsubstituted, unsaturated, monocyclic or polycyclic (fused or separate) aromatic hydrocarbon moieties. Preferred monocyclic aromatic moieties include phenyl, substituted phenyl moieties including, but not limited to, tolyl, xylyl, mesityl, cumenyl (isopropyl phenyl) and substituted phenylene derivatives including, but not limited to, benzyl, benzhydryl, cinnamyl, phenethyl, styrl and trityl. Preferred fused polycyclic moieties include substituted and unsubstituted naphthalene and anthracene moieties.

As used herein, the term "heterocyclic" is intended to embrace substituted or unsubstituted, saturated or unsaturated, monocyclic or polycyclic (fused or separate) heterocyclic moieties. Suitable non-aromatic moieties are substituted or unsubstituted piperidine, dioxanes, piperazine and pyrrolidine moieties.

As used herein, the term "heteroaryl", which is included within the scope of "heterocyclic", is intended to embrace substituted or unsubstituted, unsaturated, monocyclic or polycyclic (fused or separate) aromatic heterocyclic moieties. Preferred are substituted or unsubstituted pyridine, pyridazine, pyrimidine, pyrazine, purines, furan, pyrrole, benzofurans, indole and thiophene moieties.

As used herein, the term "aromatic" is intended to embrace a fully unsaturated, substituted or unsubstituted, cyclic moiety..

As used herein, the term "alkyl" is intended to embrace substituted or unsubstituted, straight or branched chain, saturated or unsaturated C_{1-25} alkyl, alkenyl or alkynyl moieties. Preferred are alkyl moieties such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, pentyl, isopentyl, hexyl, methylpentyl, isohexyl, heptyl, isoheptyl, octyl, isooctyl, nonyl, isononyl, decyl, isodecyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl heptadecyl, octodecyl, nonadecyl, icosyl, henicosyl, docosyl, tricosyl, tetracosyl and pentacosyl, all of which may be further substituted. Preferred alkenyl and alkynyl moieties include vinyl, ethynyl, allyl, isopropenyl, propynyl, butenyl, butynyl, pentenyl, pentynyl, hexenyl, hexynyl, heptenyl, heptynyl, octenyl, octynyl, nonenyl, nonynyl, decenyl, decynyl, undecenyl, undecynyl, dodecenyl, dodenynyl, tridecenyl, tridecynyl, tetradecenyl, tetradecynyl, pentadecenyl, pentadecynyl, hexadecenyl, hexadecynyl, heptadecenyl, heptadecynyl, octadecenyl (oleic or elaidic), octadecynyl, nonadecenyl, nonadecynyl, icosenyl, icosynyl, henicosenyl, henicosynyl, docosenyl, docosynyl, tricosenyl, tricosynyl, tetracosenyl, tetracosynyl, pentacosenyl and pentacosynyl, all of which may be further substituted.

Advantageously, R^1 and/or R^6 comprise, independently, a cyclic, preferably an aryl moiety or a heterocyclic moiety (of which thiophene, furan or pyrrole moieties are preferred) containing an electron-donating substituent to maximise extinction coefficients and to shift the maximum wavelength of absorption beyond

650nm. Alkoxy, preferably methoxy, is a preferred electron-donating substituent. Alternatively, R^1 and/or R^6 may comprise, as an electron-donor, a substituted or unsubstituted, saturated or unsaturated, straight or branched chain alkyl moiety.

Advantageously, R^2 and/or R^5 would be a heavy atom, such as a halide, most preferably chlorine, bromine or iodine, to maximise population of the triplet state of the compound due to the heavy atom affect. Alternatively, if R^2 and/or R^5 is an alkyl, cyclic or heterocyclic moiety, it may be substituted with one or more heavy atoms, for example, a halide.

Advantageously, R^3 and/or R^4 comprise a moiety, or include substituents, that would maximise localisation of the compound in the cancerous region and optimise lypophilicity of the compound. Suitable substituents for alkyl;cyclic, preferably aryl; or heterocyclic, preferably heteroaryl moieties to optimise lipophilicity include, but are not limited to, carboxylic acids (-COOH), alcohols (-OH), amines ($-NR_2$, $-NR_3$), amides (-NHCOR, -CONHR), tetrazoles ($-CN_4R$), sulphonamides ($-NHSO_2R$, $-SO_2NHR$) and esters (-COOR) in which R is a substituted or unsubstituted, straight or branched chain alkyl moiety.

Suitable substituents for alkyl, cyclic or heterocyclic moieties or, alternatively, suitable alkyl, cyclic or heterocyclic moieties to improve localisation within the cancerous region include, but are not limited to, certain carbohydrates including β-D-galactose known to play a role in tumour cell recognition (C. Kieda, and Monsigny, M. (1986). "Involvement of membrane sugar receptors and membrane glycoconjugates in the adhesion of 3LL subpopulations to cultured pulmonary cells." *Invasion Metastasis*, **6**, 347-366); certain tripeptide sequences including Arg-Gly-Asp and Asn-Gly-Arg known for their utility in targetting doxorubicin to new blood vessels within tumours (Barinaga, M. (1998) "Peptide-guided cancer drugs show promise in mice" *Science*, **279**, 323-324and Arap, W., Pasqualini, R., and Ruoslahti, E. (1998) "Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model" *Science*, **279**, 377-380.); and certain steroids

including 17β-oestradiol which may increase targetting of oestrogen receptor-positive breast cancer cells (Ferguson, A.T., Lapidus, R.G., and Davidson, N.E. (1998) "The regulation of estrogen receptor expression and function in human breast cancer" *Cancer Treat. Res.*, **94**, 255-278).

Preferably, the compounds of the present invention have an extinction coefficient of greater than 30,000 $M^{-1}cm^{-1}$ and a maximum absorbance at greater than 600nm, preferably greater than 625nm, most preferably greater than 650nm. Advantageously, the compounds of the present invention are, *in vivo*, localised within the cytoplasm, but not the nucleus, of the cells.

The present class of non-porphyrin sensitisers are a good starting point as they are amenable to modification of the phenyl rings around the periphery of the molecule to optimise their therapeutic properties.

The invention will now be further described, with reference to the following non-limiting examples:-

Example 1

Referring to the accompanying reaction scheme, synthesis of **1** was repeated using the reported three step literature procedure of Rogers (1943). In order to make the chromophore more rigid so it would have the potential to act as a PDT agent, we converted it into its BF_2 chelate **2** by reaction with boron trifluoride diethyl etherate, diisopropylamine (DIEA) in CH_2Cl_2. As the introduction of a heavy atom into a chromophore is generally accepted to facilitate enhancement of triplet state population (a requirement for singlet oxygen generation), we brominated the free β-position of both pyrrole rings of **1** with molecular bromine in toluene giving **3** in high yields. Conversion of **3** into its BF_2 chelate **4** was readily achieved using the same conditions as for **1** (see Reaction Scheme). Both **2** and **4** are metallic brown solids and have good solubility in organic solvents such as

chloroform, toluene or THF and were fully characterised by ^{1}H, ^{13}C NMR and HRMS.

Compound **2b**; ^{1}H NMR ($CDCl_3$): 3.85 (6H, s), 7.02 (6H, m), 7.45 (6H, m) 8.06 (8H, m). ^{13}C NMR ($CDCl_3$): 55.66, 114.51, 118.91, 124.42, 128.78, 129.45, 129.53, 131.83, 131.89, 131.95, 132.76, 143.40, 162.20. EI-MS: 557.

Example 2

Compound **2a** denotes Compound **2** of the Reaction Scheme, where Ar is Phenyl, whilst Compound **2b** denotes Compound **2** of the Reaction Scheme where Ar is paramethoxyphenyl. Similarly, Compounds **4a** and **4b** denote Ar as phenyl or as paramethoxyphenyl, respectively. A study of the spectroscopic properties of **2a** and **4a** in chloroform demonstrated that they have a relatively sharp absorption band of 650 - 660 nm of high molar extinction coefficients ~ 72,000, with a full width at half maximum (fwhm) of ~ 50 nm. Introduction of an electron donating alkoxy group onto the phenyl rings adjacent to the pyrrole nitrogen resulted in an increase in extinction coefficient and a significant bathochromic shift of the absorption bands for **2b** and **4b** at 688 and 679 nm respectively (Table 1, Fig 1). The absorption bands of each photosensitiser are relatively insensitive to solvent changes with solutions in water/cremophor resulting in a further bathochromic shift of ~ 10 nm (Table 1). Excitation of chloroform solutions of the **2a** and **4a** at 635 nm gave a fluorescence band at 672 and 673 nm respectively (Fig. 1, Table 2). The fluorescence quantum yield of **2a** was 0.31 and as would be expected is significantly reduced for **4a** (0.026) due to the internal heavy atom effect (Table 2). Similarly **2b** had a fluorescence quantum yield of 0.43 while **4b** was 0.28. The ability of **2** and **4** to produce singlet oxygen would be a prerequisite to them being potential PDT agents.

Table 1 Spectroscopic absorbance properties of **2** and **4**[a]

Comp	λ_{max}[b] (nm)	fwhm[b] (nm)	ε ($M^{-1}cm^{-1}$)	λ_{max}[c] (nm)
2a	650	49	72,000	658
2b	688	52	83,500	690
4a	649	47	72,700	658
4b	679	50	81,000	685

[a]Room temperature. [b]$CHCl_3$. [c]Water/cremophor.

Table 2 Extinction coefficients and fluorescence quantum yields (Φ_f)[a]

Compound	λ (nm)[b]	Φ_f[c]	λ em (nm)[d]
2a	672	0.31	683
2b	715	0.43	727
4a	673	0.026	679
4b	713	0.028	719

[a] Room temperature. [b]$CHCl_3$. [c]Relative to magnesium tetra-*tert*-butylphthalocyanine in $CHCl_3$ (Φ_f = 0.84). [d]Water/cremophor.

Single crystals X-Ray structure determination of **2b** demonstrated the conjugated nature of the chromophore with similar bond lengths in both pyrrole rings (Fig. 2).

Example 3

Cancer cellular uptake of a photosensitiser is clearly an prerequisite for it to act as a PDT agent. Delivery of our proposed PDT agents required formulation of the sensitisers in order to impart water solubility. Water / Cremophor solutions of **2a** (10^{-6} M) were added to HeLa cancer cell lines and incubated for 5, 15, 60 and 120 mins, washed with water and examined with fluorescent microscopy. Exploiting the inherent fluorescent properties of **2a**, we have observed efficient uptake and cytosolic localisation of **2a** with maximum uptake after 60 minutes (Fig 3). Dual staining of the nucleus of the cells with 4',6-diamidino-2-phenylindole (DAPI)

prior to treatment with **2a** gave good contrast imaging and confirmed localisation of **2a** primarily at the endoplasmic reticulum and not in the nucleus (Fig 4).

Example 4

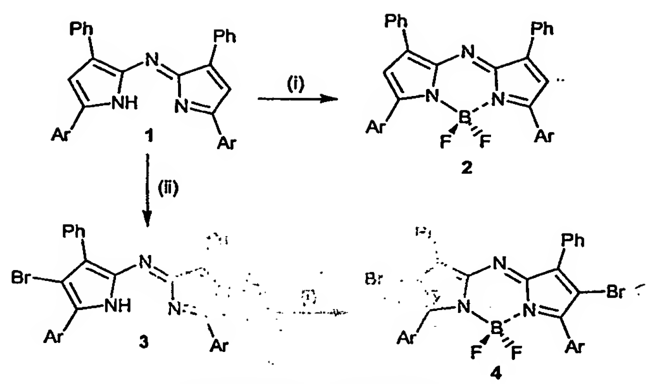
Light toxicity assays were carried out as follows:

HeLa cancer calls were exposed to **2a** in varying concentrations for 24 hours. Drug laden medium was removed and replaced with fresh medium. Cells were irradiated at constant temperature for 15 minutes with light from a 100 W or 500W halogen lamp passed through a red glass and water filter barrier, thereby ensuring cells are irradiated with light of more than 570nm. Irradiated cells were incubated for a further 24 hrs. MTT cell viability assay was performed.

A typical dose response curve is shown in Fig 5. This demonstrates an IC_{50} of $1x10^{-6}$M using the 500W light source. The poorer response for the 100 W light source demonstrates that varying the quantity of light activation has a direct effect on drug efficacy. The dark toxicity effect may be caused by the micellar delivery vehicle itself.

The present invention is not limited to the embodiments described herein, which may be amended or modified without departing from the scope of the invention.

Reaction Scheme



a Ar = Ph; b Ar = 4-$CH_3OC_6H_4$

(i) $BF_3.OEt_2$, DIEA, CH_2Cl_2, rt, 16 h;

(ii) Br_2, toluene, rt, 2 h.

Fig. 1 Normalised absorption (—) and emission spectra (....) of **2a** and electronic absorption (— — —) and fluorescence spectra (— ··—) of **4a** in $CHCl_3$ at room temperature.

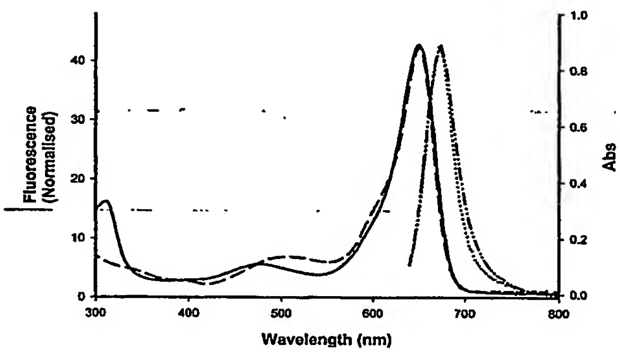


Fig. 2 X-Ray crystal structure of **4a**; crystallised from toluene/methanol bilayer (co-crystallised with molecule of toluene).

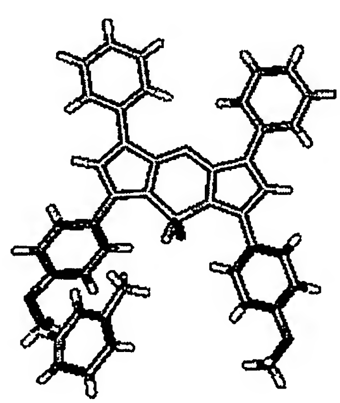


Fig 3 Cellular localisation of **2a** (light grey colour) in HeLa cancer cells visualised with fluorescent microscopy (darker grey area is the cell nucleus).

Fig 4 Cellular localisation of **2a** in HeLa cancer cells; nucleus is co-stained with DAPI (blue) and cytoplasmic localisation of **2a** (red).

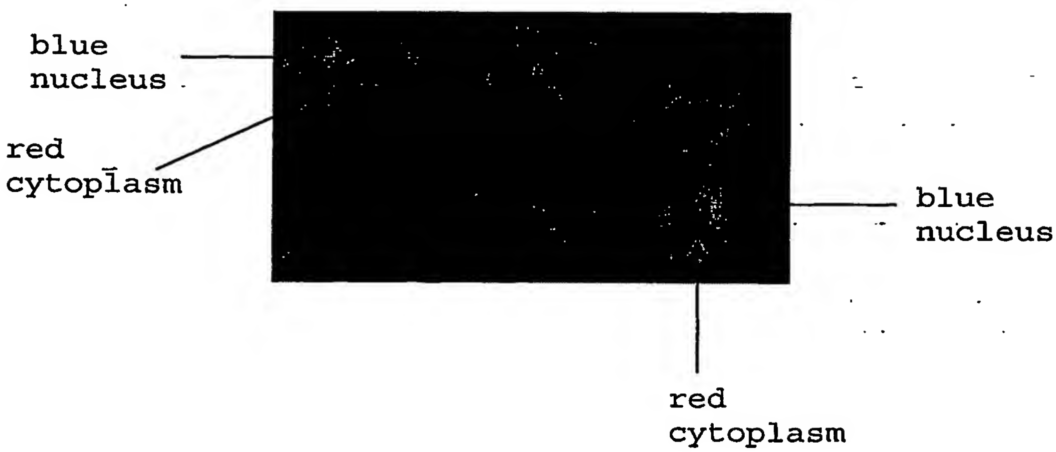


Fig 5. Dose Response Curve for 2a